

SureSelect^{XT2} Target Enrichment System for Illumina Multiplexed Sequencing

Featuring Pre-Capture
Indexing Reagents and
Protocols

Protocol

Version E0, January 2015

SureSelect platform manufactured with Agilent
SurePrint Technology

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Procedures.

Before you begin, view hands-on
videos of SureSelect procedures at
<http://www.agilent.com/genomics/protocolvideos>.



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In this Guide...

This guide describes an optimized protocol for Illumina paired-end multiplexed library preparation using the SureSelect^{XT2} Library Prep and Capture System.

This protocol is specifically optimized to enrich targeted regions of the genome from repetitive sequences and sequences unrelated to the research focus prior to sample sequencing using the Illumina platform. The SureSelect^{XT2} Library Prep and Capture System features pre-capture indexing reagents and protocols.

1 Before You Begin

This chapter contains information (such as procedural notes, safety information, required reagents and equipment) that you should read and understand before you start an experiment.

2 Sample Preparation (1 µg DNA Samples)

This chapter describes the steps to prepare index-tagged libraries for target enrichment from 1-µg gDNA samples.

3 Sample Preparation (100 ng DNA Samples)

This chapter describes the steps to prepare index-tagged libraries for target enrichment from 100-ng gDNA samples.

4 Hybridization

This chapter describes the steps to pool indexed libraries and then hybridize and capture the pooled DNA.

5 Post-Capture Sample Processing for Multiplexed Sequencing

This chapter describes the steps to amplify, purify, and assess quality and quantity of the sample libraries. Samples are pooled by mass prior to sequencing.

6 Reference

This chapter contains reference information, including component kit contents and index sequences.

What's New in Version E.0

- Support for kits supplied with either of two indexing primer configurations.

Kits with revised index configuration (typically received February 2015 or later) include indexing primers A01 through H02 provided in white-capped tubes (16 Reaction kits) or indexing primers A01 through H12 provided in a blue plate (96 Reaction kits). For kit content details see [page 76](#). For nucleotide sequences of the 8-bp indexes in this revised configuration, see [Table 38](#) on page 79.

Kits with original index configuration (typically received before February 2015), include indexing primers 1–16 or 1–96 provided in clear-capped tubes. For kit content details see [page 80](#). For nucleotide sequences of the 8-bp indexes in this original configuration, see [Table 44](#) on page 82 through [Table 49](#) on page 87.

- Support for revised Library Prep kit configuration, now including End Repair Enzyme Mix and End Repair Oligo Mix (both replacing End Repair Master Mix). For protocols for use of the revised kits, see [page 25](#) for 1 µg DNA samples or see [page 43](#) for 100 ng DNA samples. See [Table 34](#) on page 77 for updated kit contents.
- Updates to solvents used in the Sample Preparation protocol for 100 ng DNA samples. Updates include initial dilution of gDNA samples in 1X Low TE Buffer ([step 2](#) on [page 38](#)), and DNA elution from AMPure XP beads using nuclease-free water ([step 13](#) on [page 46](#) and [step 12](#) on [page 49](#)).
- Update to instructions for dilution of pre-capture indexing adaptors in the Sample Preparation protocol for 100 ng DNA samples. See [step 1](#) on [page 48](#).
- Update to instructions for washing streptavidin-coated magnetic beads. See [step 4](#) on [page 59](#).

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Before You Begin

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Make sure you read and understand the information in this chapter and have the necessary equipment and reagents listed before you start an experiment.

NOTE

Agilent cannot guarantee the SureSelect Target Enrichment kits and cannot provide technical support for the use of non-Agilent protocols or instruments to process samples for enrichment.



Overview of the Workflow

The SureSelect^{XT2} target enrichment workflow is summarized in Figure 1.

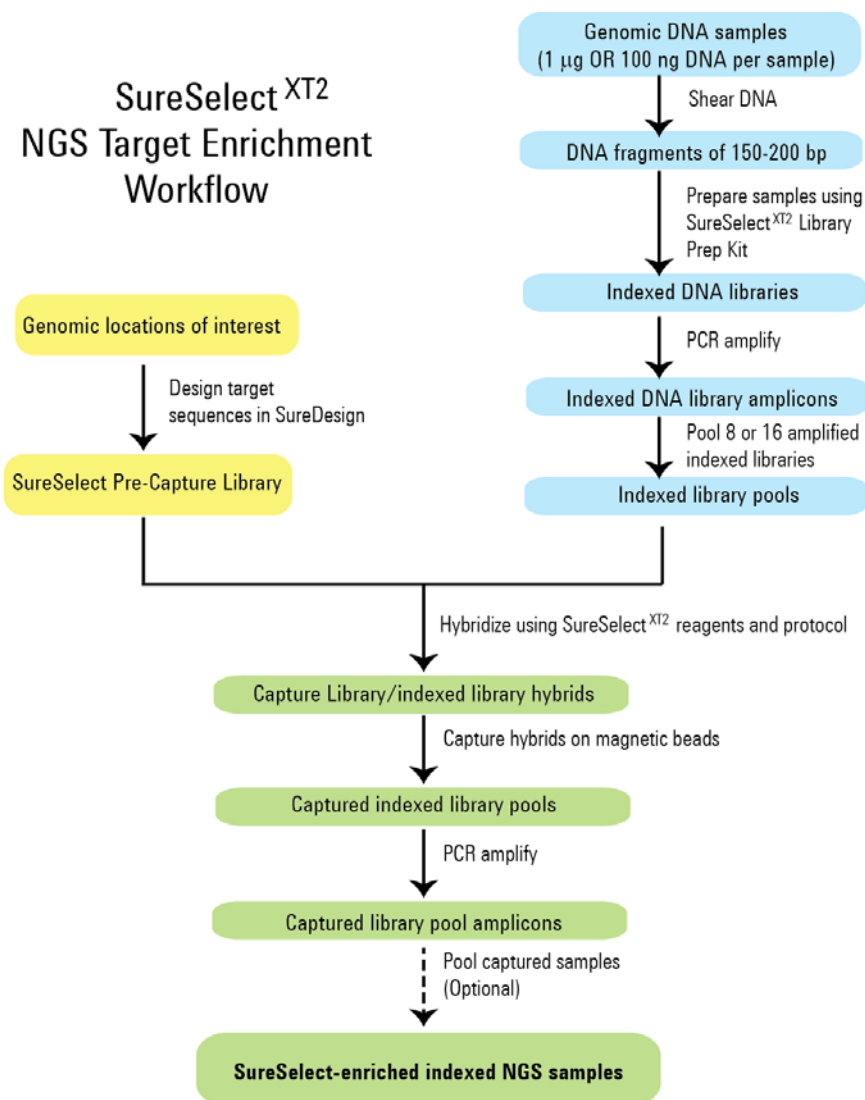


Figure 1 Overall target-enriched sequencing sample preparation workflow.

Procedural Notes

- This User Guide includes protocols for library preparation using either 1 µg DNA samples (see [Chapter 2 on page 19](#)) or 100 ng DNA samples (see [Chapter 3 on page 37](#)). Make sure that you are following the appropriate protocol for your DNA input amount. After the prepared libraries are amplified, both DNA input options use the same protocol for hybridization and post-capture processing.
- To prevent contamination of reagents by nucleases, always wear powder-free laboratory gloves and use dedicated solutions and pipettors with nuclease-free aerosol-resistant tips.
- Maintain a clean work area.
- Do not mix reactions containing gDNA on a vortex mixer. Instead, gently tap the tube with your finger to mix the sample.
- Avoid repeated freeze-thaw cycles of stock and diluted gDNA solutions. Possible stopping points, where gDNA samples may be stored overnight at 4°C, are marked in the protocol. When storing samples for >24 hours, store the samples at -20°C, but do not subject the samples to multiple freeze/thaw cycles.
- When preparing reagent stock solutions for use:
 - 1 Thaw the aliquot as rapidly as possible without heating above room temperature.
 - 2 Mix briefly on a vortex mixer, then spin in a centrifuge for 5 to 10 seconds to drive the contents off of walls and lid.
 - 3 Store vials used during an experiment on ice or in a cold block.
 - 4 Library Preparation Master Mixes should not be frozen and thawed more than five times. If you plan to use the reagents in more than five experiments, aliquot to multiple vials to minimize freeze/thaw cycles for each vial.
- In general, follow Biosafety Level 1 (BL1) safety rules.

Safety Notes

CAUTION

- Wear appropriate personal protective equipment (PPE) when working in the laboratory.

Required Reagents

Table 1 Required Reagents for SureSelect^{XT2} Target Enrichment

Description	Vendor and part number
SureSelect ^{XT2} Capture Library*	Select one library from Table 2
SureSelect ^{XT2} Reagent Kit*	Agilent
HiSeq platform (HSQ), 16 Samples	p/n G9621A
HiSeq platform (HSQ), 96 Samples	p/n G9621B
HiSeq platform (HSQ), 480 Samples	p/n G9621C
MiSeq platform (MSQ), 16 Samples	p/n G9622A
MiSeq platform (MSQ), 96 Samples	p/n G9622B
MiSeq platform (MSQ), 480 Samples	p/n G9622C
DNA 1000 Kit	Agilent p/n 5067-1504
High Sensitivity DNA Kit	Agilent p/n 5067-4626
Nuclease-free Water (not DEPC-treated)	Ambion Cat #AM9930
1X Low TE Buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA)	Life Technologies p/n 12090-015, or equivalent
Agencourt AMPure XP Kit	Beckman Coulter Genomics
5 mL	p/n A63880
60 mL	p/n A63881
450 mL	p/n A63882
Dynabeads MyOne Streptavidin T1	Life Technologies
2 mL	Cat #65601
10 mL	Cat #65602
Quant-iT dsDNA BR Assay Kit, for the Qubit fluorometer	Life Technologies
100 assays, 2-1000 ng	Cat #Q32850
500 assays, 2-1000 ng	Cat #Q32853
Qubit assay tubes	Life Technologies p/n Q32856
100% Ethanol, molecular biology grade	Sigma-Aldrich p/n E7023

* SureSelect capture libraries and reagents must be used within one year of receipt.

Table 2 SureSelect^{XT2} Capture Libraries

Capture Library	16 Samples	96 Samples	480 Samples
SureSelect ^{XT2} Clinical Research Exome*	5190-7345	5190-7346	5 × 5190-7346
SureSelect ^{XT2} Focused Exome*	5190-7797	5190-7798	5 × 5190-7798
SureSelect ^{XT2} Focused Exome Plus 1*	5190-7800	5190-7806	5 × 5190-7806
SureSelect ^{XT2} Human All Exon v5*	5190-6216	5190-6217	5 × 5190-6217
SureSelect ^{XT2} Human All Exon v5 + UTRs*	5190-6221	5190-6222	5 × 5190-6222
SureSelect ^{XT2} Human All Exon v5 + lncRNA*	5190-6452	5190-6453	5 × 5190-6453
SureSelect ^{XT2} Human All Exon v5 Plus*	5190-6219	5190-6220	5 × 5190-6220
SureSelect ^{XT2} Human All Exon v4 *	5190-4666	5190-4667	5190-4669
SureSelect ^{XT2} Human All Exon v4+ UTRs*	5190-4671	5190-4672	5190-4674
SureSelect ^{XT2} Mouse All Exon*	5190-4681	5190-4682	5190-4684
SureSelect ^{XT2} Inherited Disease*	5190-7524	5190-7525	5 × 5190-7525
SureSelect ^{XT2} Inherited Disease Plus*	5190-7527	5190-7528	5 × 5190-7528
SureSelect ^{XT2} Human DNA Kinome [†]	5190-4676	5190-4677	5190-4679
SureSelect ^{XT2} Custom 1 kb up to 499 kb [†] (reorder)	5190-4846 (5190-4851)	5190-4847 (5190-4852)	5190-4849 (5190-4854)
SureSelect ^{XT2} Custom 0.5 Mb up to 2.9 Mb [†] (reorder)	5190-4856 (5190-4861)	5190-4857 (5190-4862)	5190-4859 (5190-4864)
SureSelect ^{XT2} Custom 3 Mb up to 5.9 Mb [†] (reorder)	5190-4866 (5190-4871)	5190-4867 (5190-4872)	5190-4869 (5190-4874)
SureSelect ^{XT2} Custom 6 Mb up to 11.9 Mb [†] (reorder)	5190-4876 (5190-4881)	5190-4877 (5190-4882)	5190-4879 (5190-4884)
SureSelect ^{XT2} Custom 12 Mb up to 24 Mb [†] (reorder)	5190-4886 (5190-4891)	5190-4887 (5190-4892)	5190-4889 (5190-4894)

* Eight gDNA samples are enriched in one capture reaction after sample pooling. Capture libraries are provided for the number of capture reactions needed to enrich the indicated number of samples.

† Sixteen gDNA samples are enriched in one capture reaction after sample pooling. Capture libraries are provided for the number of capture reactions needed to enrich the indicated number of samples.

Required Equipment

Table 3 Required Equipment for SureSelect^{XT2} Target Enrichment

Description	Vendor and part number
SureCycler 8800 Thermal Cycler	Agilent p/n G8800A
96 well plate module for SureCycler 8800 Thermal Cycler	Agilent p/n G8810A
SureCycler 8800-compatible 96-well plates	Agilent p/n 410088
Optical strip caps	Agilent p/n 401425
Tube cap strips, domed	Agilent p/n 410096
Compression mats	Agilent p/n 410187
2100 Bioanalyzer Laptop Bundle	Agilent p/n G2943CA
2100 Bioanalyzer Electrophoresis Set	Agilent p/n G2947CA
Covaris Sample Preparation System, E-series or S-series	Covaris
Covaris sample holders	
96 microTUBE plate (E-series only)	Covaris p/n 520078
microTUBE for individual sample processing	Covaris p/n 520045
DNA LoBind Tubes, 1.5-mL PCR clean, 250 pieces	Eppendorf p/n 022431021 or equivalent
Centrifuge	Eppendorf Centrifuge model 5804 or equivalent
Qubit Fluorometer	Life Technologies p/n Q32857 or equivalent
Magnetic separator for 96-well plates	DynaMag-96 magnet, Life Technologies p/n 120-27 or equivalent
Nutator plate mixer	BD Diagnostics p/n 421105 or equivalent
Multichannel pipette	Pipetman or equivalent
P10, P20, P200 and P1000 pipettes	Pipetman P10, P20, P200, P1000 or equivalent
Vortex mixer	

Table 3 Required Equipment for SureSelect^{XT2} Target Enrichment

Description	Vendor and part number
Vacuum concentrator	Savant SpeedVac, model DNA120, with 96-well plate rotor, model RD2MP, or equivalent
Ice bucket	
Powder-free gloves	
Sterile, nuclease-free aerosol barrier pipette tips	
Timer	
Water bath or heat block suitable for incubation temperatures up to 65°C	

Optional Reagents and Equipment

Table 4 Optional Reagents and Equipment

Description	Vendor and part number
2200 TapeStation	Agilent p/n G2964AA or G2965AA
2200 TapeStation consumables	
D1000 ScreenTape	Agilent p/n 5067-5582
D1000 Reagents	Agilent p/n 5067-5583
High Sensitivity D1000 ScreenTape	Agilent p/n 5067-5584
High Sensitivity D1000 Reagents	Agilent p/n 5067-5585
Labnet MPS1000 Mini Plate Spinner	Labnet International p/n C1000
Magnetic separator for conical vials	DynaMag-50 magnet, Life Technologies p/n 123-02D or equivalent
QPCR NGS Library Quantification Kit (Illumina)	Agilent p/n G4880A
Mx3005P Real-Time PCR System	Agilent p/n 401449 or equivalent
Mx3000P/Mx3005P 96-well tube plates	Agilent p/n 410088 or equivalent
Mx3000P/Mx3005P optical strip caps	Agilent p/n 401425 or equivalent

1 Before You Begin

Optional Reagents and Equipment



2 Sample Preparation (1 µg DNA Samples)

- Step 1. Shear DNA 20
- Step 2. Assess quality (optional) 23
- Step 3. Repair the ends 25
- Instructions for Library Prep Kit p/n 5500-0102 or 5500-0103 26
- Step 5. Adenylate the 3' end of the DNA fragments 29
- Step 6. Ligate the pre-capture indexing adaptor 30
- Step 7. Purify the indexed DNA using AMPure XP beads 31
- Step 8. Amplify the indexed library 32
- Step 9. Purify the amplified library with AMPure XP beads 34
- Step 10. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay 35

This section contains instructions for the preparation of indexed gDNA libraries from 1 µg DNA samples. For lower input (100 ng) DNA samples, see the library preparation protocol on [page 37](#).

For each sample to be sequenced, an individual indexed library is prepared. For an overview of the SureSelect^{XT2} target enrichment workflow, see [Figure 1](#) on page 12.

The sample preparation protocol is used to prepare DNA libraries for sequencing using the Illumina paired-read sequencing platform. The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, implementation of AMPure XP beads for all purification steps, and primers used for PCR. Refer to the Illumina protocol *Preparing Samples for Multiplexed Paired-End Sequencing* (p/n1005361) or the appropriate Illumina protocol for more information.



Step 1. Shear DNA

NOTE

Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0.

For each DNA sample to be sequenced, prepare 1 library.

- 1 Use the Qubit dsDNA BR Assay to determine the concentration of your gDNA sample.

Follow the instructions for the instrument.

- 2 Dilute 1 µg of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 50 µL.

- 3 Set up the Covaris E-series or S-series instrument.

- a Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.

- b Check that the water covers the visible glass part of the tube.

- c On the instrument control panel, push the Degas button. Degas the instrument for least 2 hours before use, or according to the manufacturer's recommendations.

- d Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C.

- e *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

Refer to the Covaris instrument user guide for more details.

- 4 Put a Covaris microTube into the loading and unloading station.

Keep the cap on the tube.

NOTE

You can use the 96 microTube plate (see [Table 3](#) on page 16) for the DNA shearing step when preparing multiple gDNA samples in the same experiment.

- 5 Use a tapered pipette tip to slowly transfer the 50-µL DNA sample through the pre-split septa.

Be careful not to introduce a bubble into the bottom of the tube.

- 6 Secure the microTube in the tube holder and shear the DNA with the settings in Table 5 or Table 6, depending on the Covaris instrument SonoLab software version used.

The target DNA fragment size is 150 to 200 bp.

Table 5 Shear settings for Covaris instruments using SonoLab software version 7 or newer

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds
Bath Temperature	4° to 8° C

Table 6 Shear settings for Covaris instruments using SonoLab software prior to version 7

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° C

- 7 Put the Covaris microTube back into the loading and unloading station.
- 8 While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA.

2 Sample Preparation (1 µg DNA Samples)

Step 1. Shear DNA

- 9 Transfer each 50-µL sheared DNA sample to a separate well of a SureCycler 8800 96-well plate.

CAUTION

Use SureCycler 8800-compatible 96-well plates (see [Table 3](#) on page 16) for all SureSelect^{XT2} protocol steps done in 96-well plates.

Protocols were optimized using the specified plates; use of other plasticware may negatively impact your results and is not supported by Agilent.

Step 2. Assess quality (optional)

NOTE

You can use Agilent's 2200 TapeStation for rapid analysis of multiple samples at this step. For analysis of 1 µg sheared DNA samples, use the D1000 ScreenTape (p/n 5067-5582) and associated reagents. See the 2200 TapeStation and D1000 ScreenTape protocols for information on sample preparation and data analysis.

This step is optional.

Use a Bioanalyzer DNA 1000 chip and reagent kit for analysis of the 1 µg sheared DNA samples using the 2100 Bioanalyzer. See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

- 1 Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
- 2 Open the 2100 Expert Software (version B.02.07 or higher), turn on the 2100 Bioanalyzer and check communication.
- 3 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
- 4 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 5 Within the instrument context, choose the DNA 1000 assay from the drop down list.
- 6 Start the run. Enter sample names and comments in the Data and Assay context.
- 7 Check that the electropherogram shows an average DNA fragment size of 150 to 200 bp. A sample electropherogram is shown in [Figure 2](#).

2 Sample Preparation (1 µg DNA Samples)

Step 2. Assess quality (optional)

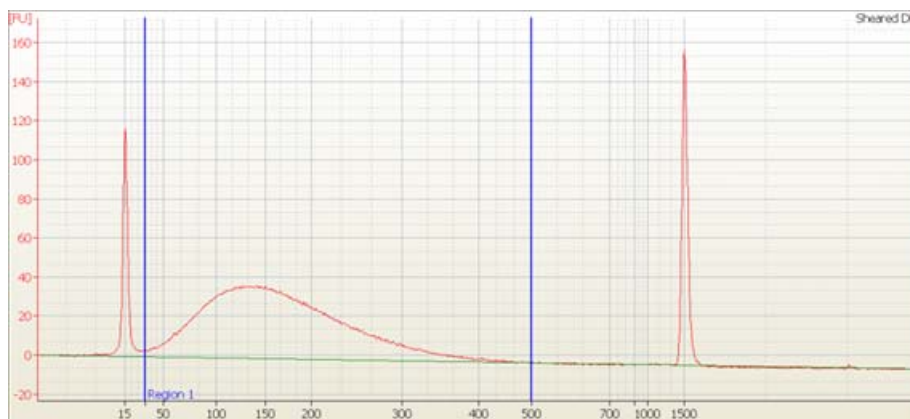


Figure 2 Analysis of sheared DNA using a DNA 1000 Bioanalyzer assay.

Step 3. Repair the ends

Use the SureSelect^{XT2} Library Prep Kit.

CAUTION

This section contains two versions of instructions for the end repair reaction. The first section covers kits supplied with Library Prep Kit p/n 5500-0130 or 5500-0131, with two separate reagent vials containing the End Repair Enzyme Mix and End Repair Oligo Mix (typically received February, 2015 or later).

The second section covers kits supplied with Library Prep Kit p/n 5500-0102 or 5500-0103, with pre-combined End Repair Master Mix (typically received before February, 2015).

Verify that you are referencing the information appropriate for your kit version before you proceed.

Hold samples on ice during the following steps.

Instructions for Library Prep Kit p/n 5500-0130 or 5500-0131

- 1 Prepare the appropriate volume of End Repair Reaction Mix, as described in [Table 7](#), on ice. Mix well on a vortex mixer.

Table 7 Preparation of SureSelect End Repair Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
SureSelect End Repair Enzyme Mix	40 µL	660 µL
SureSelect End Repair Oligo Mix	10 µL	165 µL
Total	50 µL	825 µL

- 2 To each 50-µL sheared DNA sample well, add 50 µL of the SureSelect End Repair Reaction Mix prepared in [step 1](#).
- 3 Mix well by pipetting up and down or by gentle vortexing.

2 Sample Preparation (1 µg DNA Samples)

Step 3. Repair the ends

- 4 Incubate the plate in the SureCycler thermal cycler and run the program in [Table 8](#). Do not use a heated lid.

Table 8 End-Repair Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Instructions for Library Prep Kit p/n 5500-0102 or 5500-0103

- 1 Add 50 µL of SureSelect End Repair Master Mix to each sheared DNA sample.
- 2 Mix well by pipetting up and down or by gentle vortexing.

CAUTION

SureSelect^{XT2} master mixes are viscous and thorough mixing is required to combine these mixtures with other solutions.

- 3 Incubate the plate in the SureCycler thermal cycler and run the program in [Table 8](#). Do not use a heated lid.

Table 9 End-Repair Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Step 4. Purify the sample using AMPure XP beads

- 1 Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
- 2 Prepare 400 µL of 70% ethanol per sample, plus excess, for use in [step 8](#).

NOTE

The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 1.2 mL of fresh 70% ethanol per sample.

- 3 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
- 4 Add 180 µL of homogeneous AMPure XP beads to each 100-µL end-repaired DNA library sample in the PCR plate. Pipette up and down 10 times to mix.
- 5 Incubate samples for 5 minutes at room temperature.
- 6 Put the plate into a magnetic separation device, such as the Dynal magnetic separator. Wait for the solution to clear (approximately 3 to 5 minutes).
- 7 Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

NOTE

If some magnetic beads remain suspended in solution after 5 minutes, carefully remove and discard 100 µL of cleared solution from near the bottom of the tube, and continue incubating the tube in the magnetic stand for an additional 3 minutes. After the remaining suspension has cleared, remove and discard the remaining cleared solution (approximately 180 µL) from the well.

- 8 Continue to keep the plate in the magnetic stand while you dispense 200 µL of 70% ethanol in each sample well.
Use fresh 70% ethanol for optimal results.
- 9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 10 Repeat [step 8](#) to [step 9](#) step once.

2 Sample Preparation (1 µg DNA Samples)

Step 4. Purify the sample using AMPure XP beads

- 11** Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 12** Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 5 to 10 minutes or until the residual ethanol completely evaporates.
- 13** Add 22 µL nuclease-free water to each sample well.
- 14** Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- 15** Incubate for 2 minutes at room temperature.
- 16** Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 17** Remove the cleared supernatant to a fresh SureCycler 8800 PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 5. Adenylate the 3' end of the DNA fragments

- 1 Add 20 µL of SureSelect dA-Tailing Master Mix to each end-repaired, purified DNA sample (approximately 20 µL).
- 2 Mix well by pipetting up and down or by gentle vortexing.

CAUTION

SureSelect^{XT2} master mixes are viscous and thorough mixing is required to combine these mixtures with other solutions.

- 3 Incubate the plate in the SureCycler thermal cycler and run the program in [Table 10](#). Do not use a heated lid.

Table 10 dA-Tailing Thermal Cycler Program

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	4°C	Hold

Step 6. Ligate the pre-capture indexing adaptor

See the [Reference](#) section for sequences of the index portion of the indexing adaptors that are ligated to gDNA libraries in this section.

Be sure to keep the sample plate at 4°C or on ice while doing [step 1](#) and [step 2](#) sequentially as outlined below.

- 1 Add 5 µL of SureSelect Ligation Master Mix to each A-tailed DNA sample.
- 2 Add 5 µL of the appropriate SureSelect^{XT2} Pre-capture Index solution to each sample.
- 3 Seal the wells then mix thoroughly by vortexing for 5 seconds. Briefly spin the plate, then keep the plate on ice until it is placed in the thermal cycler in [step 4](#).

CAUTION

SureSelect^{XT2} master mixes are viscous and thorough mixing is required to combine these mixtures with other solutions.

- 4 Incubate the plate in the SureCycler thermal cycler and run the program in [Table 11](#). Do not use a heated lid.

Table 11 Ligation Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	4°C	Hold

Step 7. Purify the indexed DNA using AMPure XP beads

- 1** Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
- 2** Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
- 3** Add 60 µL of homogeneous AMPure XP beads to each 50-µL indexing adaptor-ligated DNA sample in the PCR plate. Pipette up and down to mix.
- 4** Incubate samples for 5 minutes at room temperature.
- 5** Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
- 6** Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
- 7** Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
- 8** Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 9** Repeat [step 7](#) and [step 8](#) step once.
- 10** Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 11** Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 5 minutes or until the remaining ethanol completely evaporates.
- 12** Add 50 µL nuclease-free water to each sample well.
- 13** Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- 14** Incubate for 2 minutes at room temperature.
- 15** Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 16** Remove the cleared supernatant (approximately 50 µL) to a fresh SureCycler 8800 PCR plate well. You can discard the beads at this time.

2 Sample Preparation (1 µg DNA Samples)

Step 8. Amplify the indexed library

Step 8. Amplify the indexed library

This protocol uses half of the indexing adaptor-ligated library for amplification. The remainder can be saved at -20°C for future use, if needed.

CAUTION

To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

- 1 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in [Table 12](#), on ice. Mix well on a vortex mixer.

Table 12 Preparation of SureSelect Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
XT2 Primer Mix	1 µL	16.5 µL
Herculase II PCR Master Mix	25 µL	412.5 µL
Total	26 µL	429 µL

- 2 In separate wells of a SureCycler 8800 PCR plate, combine 26 µL of the amplification mixture prepared in [Table 12](#) and 24 µL of each indexed gDNA library sample.

Mix by pipetting. Change pipette tips between samples.

3 Run the program in Table 13 in a SureCycler thermal cycler.

Table 13 Pre-Capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	5	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

NOTE

Different library preparations can produce slightly different results, based on varying DNA quality. In most cases, five cycles will produce an adequate yield for subsequent capture without introducing bias or non-specific products. If yield is too low, or too high (where non-specific high molecular weight products are observed), adjust the number of cycles accordingly to amplify the remaining indexed library.

2 Sample Preparation (1 µg DNA Samples)

Step 9. Purify the amplified library with AMPure XP beads

Step 9. Purify the amplified library with AMPure XP beads

- 1 Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
- 2 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
- 3 Add 60 µL of homogeneous AMPure XP beads to each 50-µL amplified DNA sample in the PCR plate. Pipette up and down to mix.
- 4 Incubate samples for 5 minutes at room temperature.
- 5 Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
- 6 Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
- 7 Continue to keep the plate in the magnetic stand while you dispense 200 µL of freshly-prepared 70% ethanol in each sample well.
- 8 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 9 Repeat [step 7](#) and [step 8](#) step once.
- 10 Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 11 Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 5 minutes or until the residual ethanol completely evaporates.
- 12 Add 50 µL nuclease-free water to each sample well.
- 13 Seal the wells, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- 14 Incubate for 2 minutes at room temperature.
- 15 Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 16 Remove the cleared supernatant (approximately 50 µL) to a fresh SureCycler 8800 PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at 4°C overnight or at -20°C for prolonged storage.

Step 10. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay

NOTE

You can use Agilent's 2200 TapeStation for rapid analysis of multiple samples at this step. For analysis of indexed DNA amplicons prepared from 1-µg gDNA samples, use the D1000 ScreenTape (p/n 5067-5582) and associated reagents. See the 2200 TapeStation and D1000 ScreenTape protocols for information on sample preparation and data analysis.

Use a Bioanalyzer DNA 1000 chip and reagent kit for analysis of indexed DNA amplicons prepared from 1-µg gDNA samples. See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

- 1 Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
- 2 Open the 2100 Expert Software (version B.02.07 or higher), turn on the 2100 Bioanalyzer and check communication.
- 3 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 µL of each sample for the analysis.
- 4 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 5 Within the instrument context, choose the DNA 1000 assay from the drop down list.
- 6 Start the run. Enter sample names and comments in the Data and Assay context.
- 7 Verify that the electropherogram shows a distribution with an average DNA fragment size of approximately 250 to 275 bp. A sample electropherogram is shown in [Figure 3](#).
- 8 Measure the concentration of the library by integrating under the peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

After determining the DNA concentration for each sample, proceed to [“Hybridization”](#) on page 55.

2 Sample Preparation (1 µg DNA Samples)

Step 10. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay

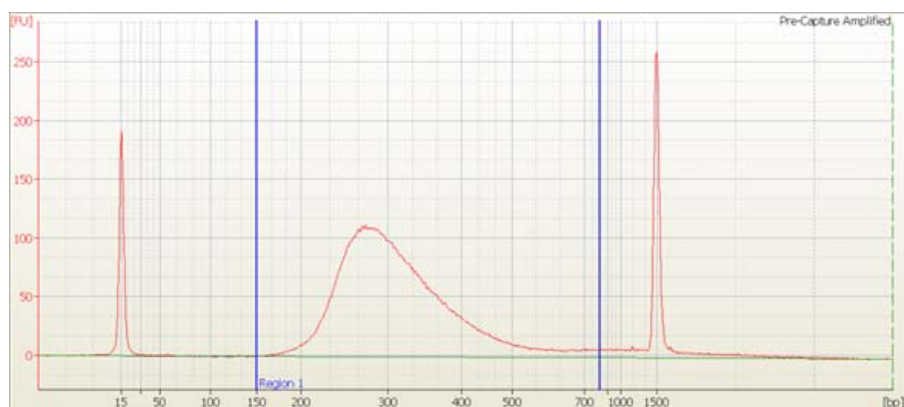


Figure 3 DNA 1000 Assay analysis of amplified library DNA prepared using the 1-µg DNA input Sample Preparation protocol.



3 Sample Preparation (100 ng DNA Samples)

- Step 1. Shear DNA 38
- Step 2. Assess quality (optional) 41
- Step 3. Repair the ends 43
- Step 4. Purify the sample using AMPure XP beads 45
- Step 5. Adenylate the 3' end of the DNA fragments 47
- Step 6. Ligate the pre-capture indexing adaptor 48
- Step 7. Purify the indexed DNA using AMPure XP beads 49
- Step 8. Amplify the indexed library 50
- Step 9. Purify the amplified library with AMPure XP beads 51
- Step 10. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay 52

This section contains instructions for the preparation of indexed gDNA libraries from 100 ng DNA samples. For higher input (1 µg) DNA samples, see the library preparation protocol on [page 19](#).

For each sample to be sequenced, an individual indexed library is prepared. For an overview of the SureSelect^{XT2} target enrichment workflow, see [Figure 1](#) on page 12.

The sample preparation protocol is used to prepare DNA libraries for sequencing using the Illumina paired-read sequencing platform. The steps in this section differ from the Illumina protocol in the use of the Covaris system for gDNA shearing, smaller target shear size, elimination of size selection by gel purification, implementation of AMPure XP beads for all purification steps, and primers used for PCR. Refer to the Illumina protocol *Preparing Samples for Multiplexed Paired-End Sequencing* (p/n1005361) or the appropriate Illumina protocol for more information.



Step 1. Shear DNA

NOTE

Make sure genomic DNA samples are of high quality with an OD 260/280 ratio ranging from 1.8 to 2.0.

For each DNA sample to be sequenced, prepare 1 library.

- 1 Use the Qubit dsDNA BR Assay to determine the concentration of your gDNA sample.

Follow the instructions for the instrument.

- 2 Dilute 100 ng of high-quality gDNA with 1X Low TE Buffer in a 1.5-mL LoBind tube to a total volume of 50 μ L.

- 3 Set up the Covaris E-series or S-series instrument.

- a Check that the water in the Covaris tank is filled with fresh deionized water to the appropriate fill line level according to the manufacturer's recommendations for the specific instrument model and sample tube or plate in use.

- b Check that the water covers the visible glass part of the tube.

- c On the instrument control panel, push the Degas button. Degas the instrument for least 2 hours before use, or according to the manufacturer's recommendations.

- d Set the chiller temperature to between 2°C to 5°C to ensure that the temperature reading in the water bath displays 5°C.

- e *Optional.* Supplement the circulated water chiller with ethylene glycol to 20% volume to prevent freezing.

Refer to the Covaris instrument user guide for more details.

- 4 Put a Covaris microTube into the loading and unloading station.

Keep the cap on the tube.

NOTE

You can use the 96 microTube plate (see [Table 3](#) on page 16) for the DNA shearing step when preparing multiple gDNA samples in the same experiment.

- 5 Use a tapered pipette tip to slowly transfer the 50- μ L DNA sample through the pre-split septa.

Be careful not to introduce a bubble into the bottom of the tube.

- 6 Secure the microTube in the tube holder and shear the DNA with the settings in Table 14 or Table 15, depending on the Covaris instrument SonoLab software version used.

The target DNA fragment size is 150 to 200 bp.

Table 14 Shear settings for Covaris instruments using SonoLab software version 7 or newer

Setting	Value
Duty Factor	10%
Peak Incident Power (PIP)	175
Cycles per Burst	200
Treatment Time	360 seconds
Bath Temperature	4° to 8° C

Table 15 Shear settings for Covaris instruments using SonoLab software prior to version 7

Setting	Value
Duty Cycle	10%
Intensity	5
Cycles per Burst	200
Time	6 cycles of 60 seconds each
Set Mode	Frequency sweeping
Temperature	4° to 7° C

- 7 Put the Covaris microTube back into the loading and unloading station.
- 8 While keeping the snap-cap on, insert a pipette tip through the pre-split septa, then slowly remove the sheared DNA.

3 Sample Preparation (100 ng DNA Samples)

Step 1. Shear DNA

- 9 Transfer each 50- μ L sheared DNA sample to a separate well of a SureCycler 8800 96-well plate.

CAUTION

Use SureCycler 8800-compatible 96-well plates (see [Table 3](#) on page 16) for all SureSelect^{XT2} protocol steps done in 96-well plates.

Protocols were optimized using the specified plates; use of other plasticware may negatively impact your results and is not supported by Agilent.

Step 2. Assess quality (optional)

NOTE

You can use Agilent's 2200 TapeStation for rapid analysis of multiple samples at this step. For analysis of 100 ng sheared DNA samples, use the High-Sensitivity D1000 ScreenTape (p/n 5067-5584) and associated reagents. See the 2200 TapeStation and High-Sensitivity D1000 ScreenTape protocols for information on sample preparation and data analysis.

This step is optional.

Use a High Sensitivity DNA Assay kit for analysis of the 100 ng sheared DNA samples using the 2100 Bioanalyzer. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com, for more information on doing this step.

- 1 Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
- 2 Open the 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit), turn on the 2100 Bioanalyzer, and check communication.
- 3 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μ L of each sheared DNA sample for the analysis.
- 4 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 5 Within the instrument context, choose the High Sensitivity DNA assay from the drop down list.
- 6 Start the run. Enter sample names and comments in the Data and Assay context.
- 7 Check that the electropherogram shows an average DNA fragment size of 150 to 200 bp. A sample electropherogram is shown in [Figure 4](#).

3 Sample Preparation (100 ng DNA Samples)

Step 2. Assess quality (optional)

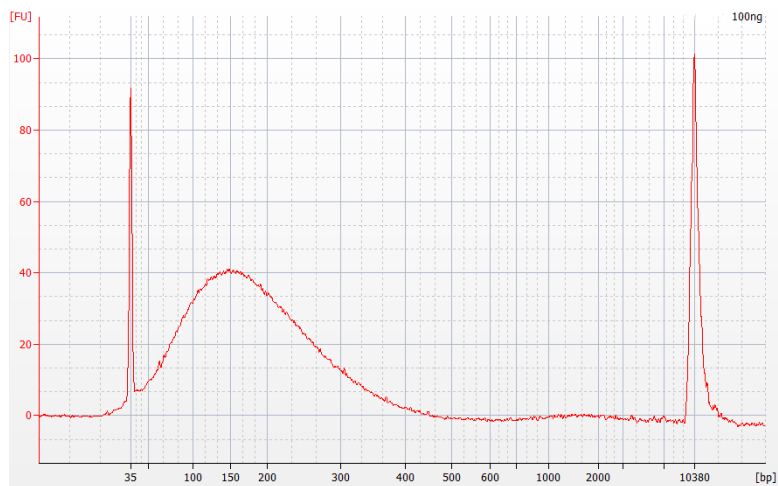


Figure 4 Analysis of 100 ng sheared DNA sample using a High-Sensitivity DNA Bioanalyzer assay.

Step 3. Repair the ends

Use the SureSelect^{XT2} Library Prep Kit.

CAUTION

This section contains two versions of instructions for the end repair reaction. The first section covers kits supplied with Library Prep Kit p/n 5500-0130 or 5500-0131, with two separate reagent vials containing the End Repair Enzyme Mix and End Repair Oligo Mix (typically received February, 2015 or later).

The second section covers kits supplied with Library Prep Kit p/n 5500-0102 or 5500-0103, with pre-combined End Repair Master Mix (typically received before February, 2015).

Verify that you are referencing the information appropriate for your kit version before you proceed.

Hold samples on ice during the following steps.

Instructions for Library Prep Kit p/n 5500-0130 or 5500-0131

- 1 Prepare the appropriate volume of End Repair Reaction Mix, as described in [Table 16](#), on ice. Mix well on a vortex mixer.

Table 16 Preparation of SureSelect End Repair Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
SureSelect End Repair Enzyme Mix	40 µL	660 µL
SureSelect End Repair Oligo Mix	10 µL	165 µL
Total	50 µL	825 µL

- 2 To each 50-µL sheared DNA sample well, add 50 µL of the SureSelect End Repair Reaction Mix prepared in [step 1](#).
- 3 Mix well by pipetting up and down or by gentle vortexing.

3 Sample Preparation (100 ng DNA Samples)

Step 3. Repair the ends

- 4 Incubate the plate in the SureCycler thermal cycler and run the program in [Table 17](#). Do not use a heated lid.

Table 17 End-Repair Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Instructions for Library Prep Kit p/n 5500-0102 or 5500-0103

- 1 Add 50 µL of SureSelect End Repair Master Mix to each sheared DNA sample.
- 2 Mix well by pipetting up and down or by gentle vortexing.

CAUTION

SureSelect^{XT2} master mixes are viscous and thorough mixing is required to combine these mixtures with other solutions.

- 3 Incubate the plate in the SureCycler thermal cycler and run the program in [Table 17](#). Do not use a heated lid.

Table 18 End-Repair Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	30 minutes
Step 2	4°C	Hold

Step 4. Purify the sample using AMPure XP beads

- 1 Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
- 2 Prepare 400 μL of 70% ethanol per sample, plus excess, for use in [step 8](#).

NOTE

The freshly-prepared 70% ethanol may be used for subsequent purification steps run on the same day. The complete Library Preparation protocol requires 1.2 mL of fresh 70% ethanol per sample.

- 3 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
- 4 Add 180 μL of homogeneous AMPure XP beads to each 100- μL end-repaired DNA library sample in the PCR plate. Pipette up and down 10 times to mix.
- 5 Incubate samples for 5 minutes at room temperature.
- 6 Put the plate into a magnetic separation device, such as the Dynal magnetic separator. Wait for the solution to clear (approximately 3 to 5 minutes).
- 7 Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.

NOTE

If some magnetic beads remain suspended in solution after 5 minutes, carefully remove and discard 100 μL of cleared solution from near the bottom of the tube, and continue incubating the tube in the magnetic stand for an additional 3 minutes. After the remaining suspension has cleared, remove and discard the remaining cleared solution (approximately 180 μL) from the well.

- 8 Continue to keep the plate in the magnetic stand while you dispense 200 μL of 70% ethanol in each sample well.
Use fresh 70% ethanol for optimal results.
- 9 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 10 Repeat [step 8](#) and [step 9](#) step once.

3 Sample Preparation (100 ng DNA Samples)

Step 4. Purify the sample using AMPure XP beads

- 11** Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 12** Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 5 to 10 minutes or until the residual ethanol completely evaporates.
- 13** Add 22 µL nuclease-free water to each sample well.
- 14** Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- 15** Incubate for 2 minutes at room temperature.
- 16** Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 17** Remove the cleared supernatant to a fresh SureCycler 8800 PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at –20°C.

Step 5. Adenylate the 3' end of the DNA fragments

- 1 Add 20 µL of SureSelect dA-Tailing Master Mix to each end-repaired, purified DNA sample (approximately 20 µL).
- 2 Mix well by pipetting up and down or by gentle vortexing.

CAUTION

SureSelect^{XT2} master mixes are viscous and thorough mixing is required to combine these mixtures with other solutions.

- 3 Incubate the plate in the SureCycler thermal cycler and run the program in [Table 19](#). Do not use a heated lid.

Table 19 dA-Tailing Thermal Cycler Program

Step	Temperature	Time
Step 1	37°C	30 minutes
Step 2	60°C	10 minutes
Step 3	4°C	Hold

NOTE

The thermal cycling program for dA-Tailing of 100 ng samples differs from the 1 µg-input dA-Tailing thermal cycling program. Be sure to include the 10-minute incubation at 60°C when preparing 100 ng DNA samples.

Step 6. Ligate the pre-capture indexing adaptor

See the [Reference](#) section for sequences of the index portion of the indexing adaptors that are ligated to gDNA libraries in this section.

Be sure to keep the sample plate at 4°C or on ice while doing [step 1](#) through [step 3](#) sequentially as outlined below.

- 1 For each of the SureSelect^{XT2} Pre-capture Index solutions to be used in the run, prepare a 1:5 dilution in nuclease-free water.
- 2 Add 5 µL of SureSelect Ligation Master Mix to each A-tailed DNA sample.
- 3 Using the diluted indexing adaptor solutions prepared in [step 1](#) above, add 5 µL of the appropriate index dilution to each sample.
- 4 Seal the wells then mix thoroughly by vortexing for 5 seconds. Briefly spin the plate, then keep the plate on ice until it is placed in the thermal cycler in [step 5](#).

CAUTION

SureSelect^{XT2} master mixes are viscous and thorough mixing is required to combine these mixtures with other solutions.

- 5 Incubate the plate in the SureCycler thermal cycler and run the program in [Table 20](#). Do not use a heated lid.

Table 20 Ligation Thermal Cycler Program

Step	Temperature	Time
Step 1	20°C	15 minutes
Step 2	4°C	Hold

Step 7. Purify the indexed DNA using AMPure XP beads

- 1** Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
- 2** Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
- 3** Add 50 μ L of homogeneous AMPure XP beads to each 50- μ L indexing adaptor-ligated DNA sample in the PCR plate. Pipette up and down to mix.
- 4** Incubate samples for 5 minutes at room temperature.
- 5** Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
- 6** Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
- 7** Continue to keep the plate in the magnetic stand while you dispense 200 μ L of freshly-prepared 70% ethanol in each sample well.
- 8** Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 9** Repeat [step 7](#) and [step 8](#) step once.
- 10** Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 11** Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 5 minutes or until the residual ethanol completely evaporates.
- 12** Add 25 μ L nuclease-free water to each sample well.
- 13** Seal the wells with strip caps, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- 14** Incubate for 2 minutes at room temperature.
- 15** Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 16** Remove the cleared supernatant (approximately 25 μ L) to a fresh SureCycler 8800 PCR plate well. You can discard the beads at this time.

3 Sample Preparation (100 ng DNA Samples)

Step 8. Amplify the indexed library

CAUTION

To avoid cross-contaminating libraries, set up PCR reactions (all components except the library DNA) in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

- 1 Prepare the appropriate volume of pre-capture PCR reaction mix, as described in [Table 21](#), on ice. Mix well on a vortex mixer.

Table 21 Preparation of SureSelect Pre-Capture PCR Reaction Mix

Reagent	Volume for 1 reaction	Volume for 16 reactions (includes excess)
XT2 Primer Mix	1 µL	16.5 µL
Herculase II PCR Master Mix	25 µL	412.5 µL
Total	26 µL	429 µL

- 2 In separate wells of a SureCycler 8800 PCR plate, combine 26 µL of the amplification mixture prepared in [Table 21](#) and 24 µL of each indexed gDNA library sample. Mix by pipetting. Change pipette tips between samples.
- 3 Run the program in [Table 22](#) in a SureCycler thermal cycler.

Table 22 Pre-Capture PCR Thermal Cycler Program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Step 9. Purify the amplified library with AMPure XP beads

- 1 Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
- 2 Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
- 3 Add 50 μ L of homogeneous AMPure XP beads to each 50- μ L amplified DNA sample in the PCR plate. Pipette up and down to mix.
- 4 Incubate samples for 5 minutes at room temperature.
- 5 Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
- 6 Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
- 7 Continue to keep the plate in the magnetic stand while you dispense 200 μ L of freshly-prepared 70% ethanol in each sample well.
- 8 Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 9 Repeat [step 7](#) and [step 8](#) step once.
- 10 Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 11 Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 5 minutes or until the residual ethanol completely evaporates.
- 12 Add 50 μ L nuclease-free water to each sample well.
- 13 Seal the plate wells, then mix well on a vortex mixer and briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- 14 Incubate for 2 minutes at room temperature.
- 15 Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 16 Remove the cleared supernatant (approximately 50 μ L) to a fresh SureCycler 8800 PCR plate well. You can discard the beads at this time.

Stopping Point If you do not continue to the next step, seal the plate and store at 4°C overnight or at -20°C for prolonged storage.

Step 10. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay

NOTE

You can use Agilent's 2200 TapeStation for rapid analysis of multiple samples at this step. For analysis of indexed DNA amplicons prepared from 100-ng gDNA samples, use the D1000 ScreenTape (p/n 5067-5582) and associated reagents. See the 2200 TapeStation and D1000 ScreenTape protocols for information on sample preparation and data analysis.

Use a Bioanalyzer DNA 1000 chip and reagent kit for analysis of indexed DNA amplicons prepared from 100-ng gDNA samples. See the *DNA 1000 Kit Guide* at www.genomics.agilent.com for more information on doing this step.

- 1 Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
- 2 Open the 2100 Expert Software (version B.02.07 or higher), turn on the 2100 Bioanalyzer and check communication.
- 3 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μ L of each sample for the analysis.
- 4 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 5 Within the instrument context, choose the DNA 1000 assay from the drop down list.
- 6 Start the run. Enter sample names and comments in the Data and Assay context.
- 7 Verify that the electropherogram shows a distribution with an average DNA fragment size of approximately 250 to 275 bp. A sample electropherogram is shown in [Figure 5](#).
- 8 Measure the concentration of the library by integrating under the peak. For accurate quantification, make sure that the concentration falls within the linear range of the assay.

After determining the DNA concentration for each sample, proceed to "[Hybridization](#)" on page 55.

Step 10. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay

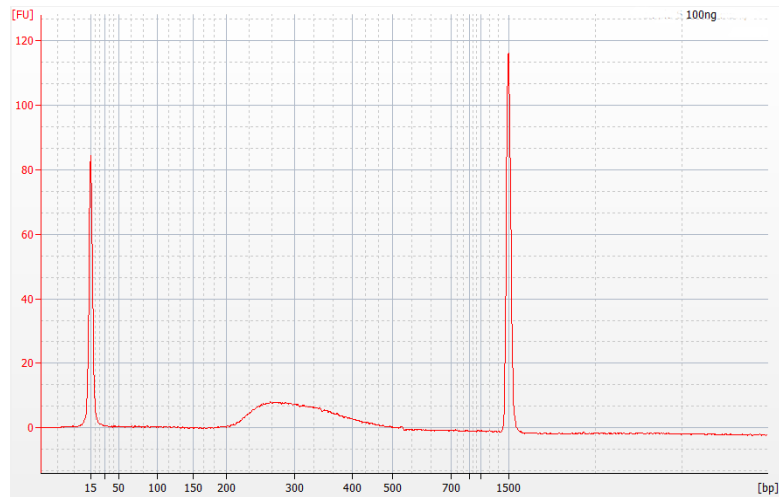


Figure 5 DNA 1000 Assay analysis of amplified library DNA prepared using the 100-ng DNA input Sample Preparation protocol.

3 Sample Preparation (100 ng DNA Samples)

Step 10. Assess quality with the 2100 Bioanalyzer DNA 1000 Assay



4 Hybridization

- Step 1. Pool indexed DNA samples for hybridization 56
- Step 2. Hybridize gDNA library pools to the SureSelect Capture Library 57
- Step 3. Prepare streptavidin-coated magnetic beads 59
- Step 4. Capture the hybridized DNA using streptavidin beads 60

This chapter describes the steps to pool indexed gDNA libraries and then hybridize the pooled gDNA libraries with a SureSelect^{XT2} Capture Library. Pools of 8 or 16 indexed samples are hybridized to the appropriate SureSelect^{XT2} Capture Library and the targeted molecules are captured for sequencing.

The size of your SureSelect^{XT2} Capture Library determines the number of indexes that may be combined for hybridization. See [Table 23](#) for the recommended number of indexes per gDNA library pool.

CAUTION

The ratio of SureSelect Capture Library to indexed gDNA library is critical for successful capture.

CAUTION

You must avoid evaporation from the small volumes of the capture during the 24 hour or greater incubation.

If you want to use a duration of hybridization >24 hours, first test the conditions. Incubate 60 µL of SureSelect XT2 Hybridization Buffer (without DNA) at 65°C for 24 hours (or longer, if applicable) as a test. Include buffer in each well that you might use, including those in the center and those on the edges. Check that you do not get extensive evaporation. Evaporation should not exceed 8 µL.



Step 1. Pool indexed DNA samples for hybridization

In this step, you pool the indexed gDNA samples, before hybridization to the SureSelect^{XT2} Capture Library.

Each hybridization reaction requires a total of 1500 ng indexed gDNA, made up of equal amounts of 8 or 16 individual libraries. See [Table 23](#) for the recommended pool composition for your SureSelect^{XT2} Capture Library.

Table 23

Capture Library size	Number of gDNA libraries per pool	Amount of each indexed library in pool
Custom Capture Libraries	16	93.75 ng
Human DNA Kinome	16	93.75 ng
Inherited Disease	8	187.5 ng
Human or Mouse All-Exon	8	187.5 ng

- 1 For each capture reaction pool, combine the appropriate volume of each indexed gDNA library sample in one well of a SureCycler 8800 PCR plate.

Each final capture reaction pool should contain 1500 ng indexed gDNA.

- 2 Use a vacuum concentrator, held at $\leq 45^{\circ}\text{C}$, to reduce the volume in each well to $<7\ \mu\text{L}$.

Avoid completely drying the sample. Over-drying the indexed library pool negatively impacts target enrichment.

- 3 Add sufficient nuclease-free water to each concentrated gDNA pool to bring the final well volume to $7\ \mu\text{L}$.
- 4 Cap the wells, then vortex the plate vigorously for 30 seconds. Spin in a centrifuge or mini-plate spinner to collect the liquid at the bottom of the wells.

Step 2. Hybridize gDNA library pools to the SureSelect Capture Library

- 1 To each 7- μ L indexed gDNA pool, add 9 μ L of SureSelect XT2 Blocking Mix. Pipette up and down to mix.
- 2 Cap the wells, then transfer the sealed plate to the thermal cycler and run the following program shown in [Table 24](#).

Use a heated lid, set at 105°C, to hold the temperature at 65°C.

Make sure that the plate is held at 65°C for at least 5 minutes before the gDNA library/Block mixtures are used in [step 7](#) below.

Table 24 Thermal cycler program used for sample denaturation prior to hybridization

Step	Temperature	Time
Step 1	95°C	5 minutes
Step 2	65°C	Hold

CAUTION

The lid of the thermal cycler is hot and can cause burns. Use caution when working near the lid.

- 3 Prepare the appropriate dilution of SureSelect RNase Block, based on the size of your capture library, according to [Table 25](#). Prepare the amount required for the number of hybridization reactions in the run, plus excess.

Table 25 Preparation of RNase Block dilution

Capture Library Size	RNase Block dilution (parts RNase Block:parts water)	Volume of dilute RNase Block Required per hybridization reaction
<3.0 Mb	10% (1:9)	5 μ L
>3.0 Mb	25% (1:3)	2 μ L

4 Hybridization

Step 2. Hybridize gDNA library pools to the SureSelect Capture Library

- 4 In a PCR plate (kept on ice), for each hybridization reaction well, combine the indicated volumes of SureSelect^{XT2} Capture Library and dilute RNase Block, according to [Table 26](#). Mix well by pipetting.

Table 26 Preparation of Capture Library/RNase Block mixture

Capture Library Size	Volume of Capture Library per hybridization reaction	Volume of dilute RNase Block per hybridization reaction
<3.0 Mb	2 µL	5 µL of 10% solution
>3.0 Mb	5 µL	2 µL of 25% solution

- 5 To each well containing 7 µL of Capture Library/RNase Block mix, add 37 µL of SureSelect XT2 Hybridization Buffer. Mix well by pipetting.

NOTE

If precipitate is present in the Hybridization Buffer, warm the solution to 65°C for 5 minutes before use.

- 6 Cap the wells, then briefly spin the plate in a centrifuge or mini-plate spinner. Keep the plate at room temperature until it is used in [step 7](#).
- 7 Maintain the gDNA pool plate at 65°C while you use a multi-channel pipette to transfer the entire 44-µL of Capture Library mixture from [step 5](#) to each sample well of the gDNA pool plate. Mix well by slowly pipetting up and down 8 to 10 times.

The hybridization reaction wells now contain approximately 60 µL.

- 8 Seal the wells with domed strip caps. Make sure that all wells are completely sealed. Place a compression mat over the PCR plate in the thermal cycler.

CAUTION

Wells must be adequately sealed to minimize evaporation, or your results can be negatively impacted.

Before you do the first experiment, make sure the plate and capping method are appropriate for the thermal cycler. Check that no more than 8 µL is lost to evaporation under the conditions used for hybridization.

- 9 Incubate the hybridization mixture for 24 hours at 65°C with a heated lid at 105°C.

Samples may be hybridized for up to 72 hours, but you must verify that the extended hybridization does not cause extensive evaporation in the sample wells.

Step 3. Prepare streptavidin-coated magnetic beads

- 1 Prewarm SureSelect XT2 Wash 2 at 65°C in a water bath or heat block for use in “Step 4. Capture the hybridized DNA using streptavidin beads”.
- 2 Vigorously resuspend the Dynabeads MyOne Streptavidin T1 magnetic beads on a vortex mixer. The magnetic beads settle during storage.
- 3 For each hybridization sample, add 50 µL of the resuspended beads to wells of a SureCycler 8800 PCR plate.
- 4 Wash the beads:
 - a Add 200 µL of SureSelect XT2 Binding Buffer.
 - b Mix by pipetting up and down until the beads are fully resuspended.
 - c Put the plate into a magnetic separator device.
 - d Wait for the solution to clear, then remove and discard the supernatant.
 - e Repeat step a through step d for a total of 3 washes.
- 5 Resuspend the beads in 200 µL of SureSelect XT2 Binding Buffer.

NOTE

For runs that include multiple sample capture wells, the streptavidin beads may be batch-washed in an Eppendorf tube or conical vial. Start the batch-washing procedure using excess bead solution. After resuspending the washed beads in the appropriate volume of SureSelect Binding Buffer, aliquot 200 µL of the washed beads to each well to be used for hybridization capture.

Step 4. Capture the hybridized DNA using streptavidin beads

- 1 Estimate and record the volume of hybridization solution that remains after the 24 hour incubation.

CAUTION

Excessive evaporation, such as when less than 52 μL remains after hybridization, can indicate suboptimal capture performance.

- 2 Maintain the hybridization plate at 65°C while you use a multichannel pipette to transfer the entire volume (approximately 60 μL) of each hybridization mixture to the plate wells containing 200 μL of washed streptavidin beads.

Mix well by slowly pipetting up and down 3 to 5 times.

- 3 Cap the wells, then incubate the capture plate on a Nutator mixer or equivalent for 30 minutes at room temperature.

Make sure the samples are properly mixing in the wells.

- 4 Briefly spin the plate in a centrifuge or mini-plate spinner.
- 5 Put the plate in a magnetic separator to collect the beads from the suspension. Remove and discard the supernatant.
- 6 Resuspend the beads in 200 μL of SureSelect XT2 Wash 1. Mix by pipetting up and down until the beads are fully resuspended.
- 7 Briefly spin in a centrifuge or mini-plate spinner.
- 8 Put the plate in the magnetic separator.
- 9 Wait for the solution to clear, then remove and discard the supernatant.

CAUTION

It is important to maintain bead suspensions at 65°C during the washing procedure below to ensure specificity of capture.

Make sure that the SureSelect XT2 Wash 2 is pre-warmed to 65°C before use.

Do not use a tissue incubator, or other devices with significant temperature fluctuations, for the incubation steps.

Step 4. Capture the hybridized DNA using streptavidin beads

10 Wash the beads with SureSelect XT2 Wash 2:

- a** Resuspend the beads in 200 μ L of 65°C prewarmed SureSelect XT2 Wash 2. Pipette up and down until the beads are fully resuspended.
- b** Incubate the sample plate for 5 minutes at 65°C on the SureCycler thermal cycler.
- c** Briefly spin the plate in a centrifuge or mini-plate spinner.
- d** Put the plate in the magnetic separator.
- e** Wait for the solution to clear, then remove and discard the supernatant.
- f** Repeat [step a](#) through [step e](#) for a total of 6 washes.
Make sure all of the wash buffer has been removed during the final wash.

11 Mix the beads in each well with 30 μ L of nuclease-free water on a vortex mixer for 5 seconds to resuspend the beads.**NOTE**

Captured DNA is retained on the streptavidin beads during the post-capture amplification step.

Stopping Point If you do not continue to the next step, seal the plate and store at 4°C overnight or at -20°C for prolonged storage.

4 Hybridization

Step 4. Capture the hybridized DNA using streptavidin beads



5 Post-Capture Sample Processing for Multiplexed Sequencing

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- Step 6. Optional: Pool captured libraries for sequencing 73

This chapter describes the steps to amplify, purify, and assess quality and quantity of the captured libraries. Post-capture dilution and optional pooling instructions are provided to prepare the indexed samples for multiplexed sequencing.



5 Post-Capture Sample Processing for Multiplexed Sequencing

Step 1. Amplify the captured libraries

Step 1. Amplify the captured libraries

In this step, the SureSelect-enriched indexed library DNA pools are PCR amplified. The protocol uses half of the bead-bound captured library pool for amplification. The remainder can be saved at -20°C for future use, if needed.

CAUTION

To avoid cross-contaminating libraries, set up PCR master mixes in a dedicated clean area or PCR hood with UV sterilization and positive air flow.

Prepare 1 amplification reaction for each captured library pool.

- 1 Prepare the appropriate volume of PCR reaction mixture, according to [Table 27](#). Mix well using a vortex mixer and keep on ice.

Table 27 Preparation of Post-Capture PCR Reaction Mix

SureSelect ^{XT2} Reagent	Volume for 1 Amplification Reaction	Volume for 12 Amplification Reactions (includes excess)
Nuclease-free water	9 μL	112.5 μL
Herculase II Master Mix	25 μL	312.5 μL
XT2 Primer Mix	1 μL	12.5 μL
Total Volume	35 μL	437.5 μL

- 2 For each amplification reaction, place 35 μL of the PCR reaction mixture from [step 1](#) in the wells of a SureCycler 8800 PCR plate.
- 3 Pipette each of the bead-bound captured library pool samples up and down to ensure that the bead suspension is homogeneous.
- 4 Add 15 μL of each captured library pool bead suspension to the appropriate PCR reaction mixture well. Mix thoroughly by pipetting until the bead suspension is homogeneous. Proceed immediately to thermal cycling in [step 5](#).

- 5 Place the plate in a thermal cycler and run the PCR amplification program shown in [Table 28](#) using the cycle number specified in [Table 29](#).

Table 28 Post-Capture PCR cycling program

Segment	Number of Cycles	Temperature	Time
1	1	98°C	2 minutes
2	8-11 see Table 29	98°C	30 seconds
		60°C	30 seconds
		72°C	1 minute
3	1	72°C	10 minutes
4	1	4°C	Hold

Table 29 Recommended cycle number based on SureSelect Capture Library size

Size of SureSelect Capture Library	Cycles
1 to 499 kb	12 to 14 cycles
0.5 to 1.49 Mb	9 to 11 cycles
> 1.5 Mb	8 to 10 cycles
All Exon	8 to 10 cycles

NOTE

Amplify the captured DNA using a minimal number of PCR cycles. If yield is too low, or too high (where non-specific high molecular weight products are observed), adjust the number of cycles accordingly with the remaining captured DNA template.

Step 2. Purify the amplified captured libraries using AMPure XP beads

- 1** Let the AMPure XP beads come to room temperature for at least 30 minutes. *Do not freeze the beads at any time.*
- 2** Prepare 400 µL of 70% ethanol per sample, plus excess, for use in [step 9](#).
- 3** Mix the bead suspension well so that the reagent appears homogeneous and consistent in color.
- 4** Add 90 µL of the homogeneous AMPure XP bead suspension to each sample well of the PCR plate, containing the 50-µL amplified captured library samples (also containing streptavidin beads used for capture).
- 5** Mix well on a vortex mixer. Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- 6** Incubate for 5 minutes at room temperature.
- 7** Put the plate into a magnetic separation device. Wait for the solution to clear (approximately 3 to 5 minutes).
- 8** Keep the plate in the magnetic stand. Carefully remove and discard the cleared solution from each well. Do not touch the beads while removing the solution.
- 9** Continue to keep the plate in the magnetic stand while you dispense 200 µL of 70% ethanol into each sample well.
Use fresh 70% ethanol for optimal results.
- 10** Wait for 1 minute to allow any disturbed beads to settle, then remove the ethanol.
- 11** Repeat [step 9](#) and [step 10](#) step once.
- 12** Seal the wells with strip caps, then briefly spin the plate to collect the residual ethanol. Return the plate to the magnetic stand for 30 seconds. Remove the residual ethanol with a P20 pipette.
- 13** Dry the samples on the SureCycler thermal cycler, set to hold samples at 37°C, for 5 minutes or until the residual ethanol completely evaporates.
- 14** Add 30 µL nuclease-free water to each sample well then mix well on a vortex mixer. Briefly spin the plate in a centrifuge or mini-plate spinner to collect the liquid.
- 15** Incubate for 2 minutes at room temperature.

Step 2. Purify the amplified captured libraries using AMPure XP beads

- 16** Put the plate in the magnetic stand and leave for 2 to 3 minutes, until the solution is clear.
- 17** Remove the cleared supernatant (approximately 30 μ L) to a fresh tube or plate well. You can discard the beads at this time.
- 18** Remove 1 μ L of the purified captured library pool from the sample and combine with 9 μ L of 1 X Low TE Buffer for Bioanalyzer analysis.

Stopping Point If you do not continue to the next step, seal the plate and store at 4°C overnight or at –20°C for prolonged storage.

Step 3. Assess quality with the 2100 Bioanalyzer High Sensitivity DNA assay

NOTE

You can use Agilent's 2200 TapeStation for rapid analysis of multiple samples at this step. For analysis of captured DNA, use the High-Sensitivity D1000 ScreenTape (p/n 5067-5584) and associated reagents. See the 2200 TapeStation and High-Sensitivity D1000 ScreenTape protocols for information on sample preparation and data analysis.

Use a High Sensitivity DNA Assay kit to assess sample quality and quantity using the 2100 Bioanalyzer. See the *High Sensitivity DNA Kit Guide* at www.genomics.agilent.com, for more information on doing this step.

NOTE

Prior to Bioanalyzer analysis, dilute each amplified captured library sample ten- fold in TE buffer, as described in [step 18](#) of the previous section.

- 1 Check that the 2100 Bioanalyzer electrodes have been cleaned as instructed in the reagent kit guide.
- 2 Open the 2100 Expert Software (version B.02.07 or higher required to run the High Sensitivity Kit), turn on the 2100 Bioanalyzer, and check communication.
- 3 Prepare the chip, samples and ladder as instructed in the reagent kit guide, using 1 μ L of diluted captured library samples for the analysis.
- 4 Load the prepared chip into the 2100 Bioanalyzer and start the run within five minutes after preparation.
- 5 Within the instrument context, choose the High Sensitivity DNA assay from the drop down list.
- 6 Start the run. Enter sample names and comments in the Data and Assay context.
- 7 Verify the results. Check that the electropherogram shows a distribution with a fragment size peak between approximately 275 to 300 bp. A sample electropherogram is shown in [Figure 6](#).
- 8 Determine the concentration of each captured indexed library pool by integration under the peak in the electropherogram.

Step 3. Assess quality with the 2100 Bioanalyzer High Sensitivity DNA assay

If the yield is too low or non-specific peaks are observed in the electropherogram, repeat the PCR with more or fewer cycles. The goal is to minimize cycles, while you produce enough library for application to the flow cell.

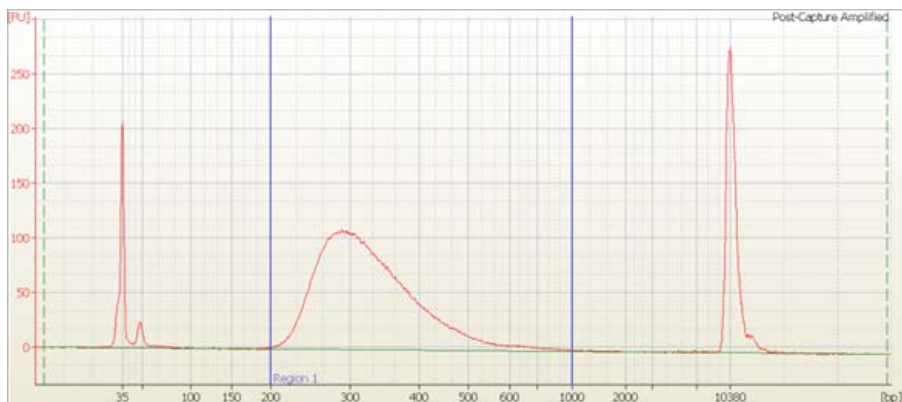


Figure 6 Analysis of amplified captured DNA using the High Sensitivity DNA Assay.

Step 4. Prepare samples for multiplexed sequencing

The final SureSelect^{XT2}-enriched samples contain pools of either 8 or 16 indexed libraries, based on the Capture Library size and resulting pre-capture pooling strategy.

The total number of indexed libraries that may be multiplexed in a single sequencing lane is determined by the output specifications of the platform used, together with the amount of sequencing data required for your Capture Library. See [Table 30](#) for sequence data requirement guidelines based on Capture Library size. Calculate the number of indexes that can be combined per lane, according to the capacity of your platform.

Table 30 Sequencing data requirement guidelines

Capture Library Size	Recommended Sequencing Data per Indexed Library	Recommended Sequencing Data per Pre-capture Pool
1 kb up to 499 kb	0.1 to 50 Mb*	1.6 to 800 Mb (16-index pools)
0.5 Mb up to 2.9 Mb	50 to 290 Mb*	0.8 to 4.64 Gb (16-index pools)
3 Mb up to 5.9 Mb	300 to 590 Mb*	4.8 to 9.44 Gb (16-index pools)
6 Mb up to 11.9 Mb	600 to 1190 Mb*	9.6 to 19 Gb (16-index pools)
12 Mb up to 24 Mb	1.2 to 2.4 Gb*	19.2 to 38.4 Gb (16-index pools)
Human DNA Kinome	320 Mb	5.12 Gb (16-index pools)
Human All Exon v5	4 Gb	32 Gb (8-index pools)
Human All Exon v5 + UTRs	6 Gb	48 Gb (8-index pools)
Human All Exon v4	4 Gb	32 Gb (8-index pools)
Human All Exon v4 + UTRs	6 Gb	48 Gb (8-index pools)
Human All Exon 50 Mb	5 Gb	40 Gb (8-index pools)
Mouse All Exon	5 Gb	40 Gb (8-index pools)

* For custom libraries, Agilent recommends analyzing 100X amount of sequencing data compared to the Capture Library size for each sample. Pool samples according to your expected sequencing output.

When appropriate for your sequencing platform, the 8-plex or 16-plex samples may be further multiplexed by post-capture pooling, using the guidelines provided in [“Step 6. Optional: Pool captured libraries for sequencing”](#) on page 73. Prior to post-capture pooling, the DNA

concentration of each sample may be accurately determined as described in “Step 5. Optional: Quantify captured library pools by QPCR” on page 72.

Sequencing run setup guidelines

If samples will not be further combined in post-capture pools, proceed to cluster amplification using the Illumina Paired-End Cluster Generation Kit; refer to the manufacturer’s instructions for this step. The optimal seeding concentration for cluster amplification from SureSelect^{XT2} DNA libraries is approximately 6 to 8 pM.

NOTE

The optimal seeding concentration may vary, depending on the method used for library quantification and fragment size distribution.

For sequencing exome captures, Agilent recommends a cluster density of approximately 800K to 900K clusters/mm³. If sequencing smaller captures, with or without pooling, the cluster density can be adjusted appropriately for improved base quality and amount of coverage desired.

Sequencing runs must be set up to perform an 8-nt index read. For the HiSeq platform, use the *Cycles* settings shown in Table 31. Cycle number settings can be specified on the *Run Configuration* screen of the instrument control software interface after choosing *Custom* from the index type selection buttons.

For complete index sequence information, see the [Reference](#) chapter starting on page 75.

Table 31 HiSeq platform Run Configuration screen Cycle Number settings *

Run Segment	Cycle Number
Read 1	100
Index 1 (i7)	9
Index 2 (i5)	0
Read 2	100

* Settings apply to v3.0 SBS chemistry.

Step 5. Optional: Quantify captured library pools by QPCR

For accurate determination of the DNA concentration in each captured library pool, use the QPCR NGS Library Quantification Kit (for Illumina).

Refer to the protocol that is included with the QPCR NGS Library Quantification Kit (p/n G4880A) for more details to do this step.

- 1 Prepare a standard curve using the quantification standard included in the kit, according to the instructions provided in the user guide.
- 2 Dilute each captured library pool such that it falls within the range of the standard curve.

Typically this corresponds to approximately a 1:1000 to 1:10,000 dilution of the captured DNA.

- 3 Prepare the QPCR master mix with Illumina adaptor-specific PCR primers according to instructions provided in the kit.
- 4 Add an aliquot of the master mix to PCR tubes and add template.
- 5 On a QPCR system, such as the Mx3005p, run the thermal profile outlined in the QPCR NGS Library Quantification kit user guide. Use the SYBR Green instrument setting.
- 6 Use the standard curve to determine the concentration of each unknown captured library pool, in nM.

The concentration will be used to accurately pool samples for multiplexed sequencing.

NOTE

In most cases, the cycle numbers in [Table 28](#) will produce an adequate yield for sequencing without introducing bias or non-specific products. If yield is too low or non-specific products are observed, adjust the number of cycles accordingly with the remaining captured DNA template.

Step 6. Optional: Pool captured libraries for sequencing

See [Table 32](#) on page 74 for post-capture pooling guidelines, based on your SureSelect^{XT2} Capture Library size and sequencing design. Pooling instructions are provided below.

- 1 Combine the capture pools such that each index-tagged sample is present in equimolar amounts in the final sequencing sample pool. For each final pool, use the formula below to determine the amount of each capture pool to use.

$$\text{Volume of capture pool} = \frac{V(f) \times C(f)}{\# \times C(i)} \quad \text{where}$$

where $V(f)$ is the final desired volume of the sequencing sample pool,
 $C(f)$ is the desired final concentration of all the DNA in the pool
 $\#$ is the number of capture pool samples to be combined, and
 $C(i)$ is the initial concentration of each capture pool sample.

- 2 Adjust the final volume of the pooled library to the desired final concentration.
 - If the final volume of the combined index-tagged samples is less than the desired final volume, $V(f)$, add Low TE to bring the volume to the desired level.
 - If the final volume of the combined index-tagged samples is greater than the final desired volume, $V(f)$, lyophilize and reconstitute to the desired volume.

[Table 32](#) shows an example of the amount of 2 capture pool samples (of different concentrations) and Low TE needed for a final volume of 20 μL at 10 nM final DNA concentration.

5 Post-Capture Sample Processing for Multiplexed Sequencing

Step 6. Optional: Pool captured libraries for sequencing

Table 32 Example of capture pool volume calculations for a 20- μ L final sequencing sample pool containing 10 nM DNA

Component	V(f)	C(i)	C(f)	#	Volume to use (μ L)
Sample 1	20 μ L	20 nM	10 nM	6	5.0
Sample 2	20 μ L	15 nM	10 nM	6	6.7
Low TE					8.3

3 If you store the library before sequencing, add Tween 20 to 0.1% v/v and store at -20°C short term.

4 Proceed to template denaturation and flow cell preparation. Refer to the appropriate Illumina protocol.

Specific library pool dilution and processing can vary based on the flow cell capacity and analysis pipeline versions being used. Refer to the appropriate Illumina user guide for instructions.

See [page 71](#) for sequencing run setup guidelines for SureSelect^{XT2} libraries.



6 Reference

Reference Information for Kits with Revised Index Configuration (indexing primers in white-capped tubes or blue plate) [76](#)

Reference Information for Kits with Original Index Configuration (indexing primers in clear-capped tubes) [80](#)

This chapter contains reference information, including component kit contents and index sequences.

CAUTION

This chapter contains two sets of kit content and index sequence information.

The first section covers revised configuration kits supplied with Library Prep Kit p/n 5500-0130 or 5500-0131 (typically received February, 2015 or later). For these kits, indexing primers are supplied in white-capped tubes or a blue plate and end-repair reagents are supplied in two separate vials.

The second section covers original configuration kits supplied with Library Prep Kit p/n 5500-0102 or 5500-0103 (typically received before February, 2015). For these kits, indexing primers are supplied in clear-capped tubes and end-repair reagents are supplied in a single master mix vial.

Verify that you are referencing the information appropriate for your kit version before you proceed.



Reference Information for Kits with Revised Index Configuration (indexing primers in white-capped tubes or blue plate)

Use the reference information in this section if your kit includes **Library Prep Kit p/n 5500-0130 or 5500-0131**. If your kit does not include one of these component kits, see [page 80](#) for kit content and indexing primer information.

Kit Contents

SureSelect^{XT2} Reagent Kits contain the following component kits:

Table 33 SureSelect^{XT2} Reagent Kit Content-Revised Index Configuration

Component Kits*	Storage Condition	16 Samples [†]	96 Samples [‡]	480 Samples ^{**}
SureSelect XT2 Library Prep Kit, ILM	–20°C	5500-0130	5500-0131	5 x 5500-0131
SureSelect XT2 Pre-Capture Box 1	Room Temperature	5190-4076	5190-4076	5190-4077
SureSelect XT2 Pre-Capture ILM Module Box 2	–20°C	5190-4461	5190-3940	5190-3942

* See [Table 34](#) through [Table 36](#) for a list of reagents included in each component kit.

† Kits contain reagents to prepare indexed libraries from 16 gDNA samples and to enrich the samples in 1 or 2 hybridization and capture reactions (as appropriate for the specific capture library size and sample pooling format).

‡ Kits contain reagents to prepare indexed libraries from 96 gDNA samples and to enrich the samples in 6 or 12 hybridization and capture reactions (as appropriate for the specific capture library size and sample pooling format).

** Kits contain reagents to prepare indexed libraries from 480 gDNA samples and to enrich the samples in 30 or 60 hybridization and capture reactions (as appropriate for the specific capture library size and sample pooling format).

NOTE

SureSelect capture libraries and reagents must be used within one year of receipt.

The contents of each of the component kits listed in [Table 33](#) are described in the tables below.

Table 34 SureSelect XT2 Library Prep Kit, ILM Content-Revised Configuration

Kit Component	16 Sample Kit	96 or 480 Sample Kit
SureSelect End Repair Enzyme Mix	tube with orange cap	bottle
SureSelect End Repair Oligo Mix	tube with green cap	tube with green cap
SureSelect dA-Tailing Master Mix	tube with yellow cap	bottle
SureSelect Ligation Master Mix	tube with purple cap	tube with purple cap
SureSelect Herculanase II Master Mix	tube with red cap	bottle
XT2 Primer Mix	tube with clear cap	tube with clear cap
SureSelect XT2 Pre-Capture Indexes*	Indexes A01 through H02, provided in 16 tubes with white caps	Indexes A01 through H12, provided in blue 96-well plate†

* See [Table 38](#) on page 79 for index sequences.

† See [Table 37](#) on page 78 for a plate map.

Table 35 SureSelect XT2 Pre-Capture Box 1 Content

Kit Component	16 Sample Kit	96 Sample Kit	480 Sample Kit
SureSelect XT2 Binding Buffer	bottle	bottle	bottle
SureSelect XT2 Wash 1	bottle	bottle	bottle
SureSelect XT2 Wash 2	bottle	bottle	bottle

Table 36 SureSelect XT2 Pre-Capture ILM Module Box 2 Content

Kit Component	16 Sample Kit	96 Sample Kit	480 Sample Kit
SureSelect XT2 Blocking Mix	tube with blue cap	tube with blue cap	tube with blue cap
SureSelect XT2 Hybridization Buffer	tube with yellow cap	tube with yellow cap	bottle
SureSelect RNase Block	tube with purple cap	tube with purple cap	tube with purple cap

Table 37 Plate map for Indexes A01 through H12 provided in blue plate in Library Prep kit p/n 5500-0131

	1	2	3	4	5	6	7	8	9	10	11	12
A	A01	A02	A03	A04	A05	A06	A07	A08	A09	A10	A11	A12
B	B01	B02	B03	B04	B05	B06	B07	B08	B09	B10	B11	B12
C	C01	C02	C03	C04	C05	C06	C07	C08	C09	C10	C11	C12
D	D01	D02	D03	D04	D05	D06	D07	D08	D09	D10	D11	D12
E	E01	E02	E03	E04	E05	E06	E07	E08	E09	E10	E11	E12
F	F01	F02	F03	F04	F05	F06	F07	F08	F09	F10	F11	F12
G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
H	H01	H02	H03	H04	H05	H06	H07	H08	H09	H10	H11	H12

Nucleotide Sequences of SureSelect^{XT2} Indexes A01 to H12

Each index is 8 nt in length. Sequencing protocols must be run using the third-read protocol that is also used for 9-bp indexes. See [page 71](#) for additional sequencing run setup information

Table 38 SureSelect^{XT2} Indexes, for indexing primers provided in blue 96-well plate

Index	Sequence		Index	Sequence		Index	Sequence		Index	Sequence
A01	ATGCCTAA		A04	AACTCACC		A07	ACGTATCA		A10	AATGTTGC
B01	GAATCTGA		B04	GCTAACGA		B07	GTCTGTCA		B10	TGAAGAGA
C01	AACGTGAT		C04	CAGATCTG		C07	CTAAGGTC		C10	AGATCGCA
D01	CACTTCGA		D04	ATCCTGTA		D07	CGACACAC		D10	AAGAGATC
E01	GCCAAGAC		E04	CTGTAGCC		E07	CCGTGAGA		E10	CAACCACA
F01	GACTAGTA		F04	GCTCGGTA		F07	GTGTTCTA		F10	TGGAACAA
G01	ATTGGCTC		G04	ACACGACC		G07	CAATGGAA		G10	CCTCTATC
H01	GATGAATC		H04	AGTCACTA		H07	AGCACCTC		H10	ACAGATTC
A02	AGCAGGAA		A05	AACGCTTA		A08	CAGCGTTA		A11	CCAGTTCA
B02	GAGCTGAA		B05	GGAGAACA		B08	TAGGATGA		B11	TGGCTTCA
C02	AAACATCG		C05	CATCAAGT		C08	AGTGGTCA		C11	CGACTGGA
D02	GAGTTAGC		D05	AAGGTACA		D08	ACAGCAGA		D11	CAAGACTA
E02	CGAACTTA		E05	CGCTGATC		E08	CATACCAA		E11	CCTCCTGA
F02	GATAGACA		F05	GGTGCGAA		F08	TATCAGCA		F11	TGGTGGTA
G02	AAGGACAC		G05	CCTAATCC		G08	ATAGCGAC		G11	AACAACCA
H02	GACAGTGC		H05	CTGAGCCA		H08	ACGCTCGA		H11	AATCCGTC
A03	ATCATTCC		A06	AGCCATGC		A09	CTCAATGA		A12	CAAGGAGC
B03	GCCACATA		B06	GTACGCAA		B09	TCCGTCTA		B12	TTCACGCA
C03	ACCACTGT		C06	AGTACAAG		C09	AGGCTAAC		C12	CACCTTAC
D03	CTGGCATA		D06	ACATTGGC		D09	CCATCCTC		D12	AAGACGGA
E03	ACCTCCAA		E06	ATTGAGGA		E09	AGATGTAC		E12	ACACAGAA
F03	GCGAGTAA		F06	GTCGTAGA		F09	TCTTCACA		F12	GAACAGGC
G03	ACTATGCA		G06	AGAGTCAA		G09	CCGAAGTA		G12	AACCGAGA
H03	CGGATTGC		H06	CCGACAAC		H09	CGCATACA		H12	ACAAGCTA

Reference Information for Kits with Original Index Configuration (indexing primers in clear-capped tubes)

Use the reference information in this section if your kit includes **Library Prep Kit p/n 5500-0102 or 5500-0103 and Pre-Capture Index Kit p/n 5190-3933, 5190-3936 or 5190-3937**. If your reagent kit does not include these component kits, see [page 76](#) for kit content and indexing primer information.

Kit Contents

SureSelect^{XT2} Reagent Kits contain the following component kits:

Table 39 SureSelect^{XT2} Reagent Kit Content-Original Index Configuration

Component Kits*	Storage Condition	16 Samples [†]	96 Samples [‡]	480 Samples ^{**}
SureSelect XT2 Library Prep Kit, ILM	–20°C	5500-0102	5500-0103	5 x 5500-0103
SureSelect XT2 Pre-Capture Indexes, ILM ^{††}	–20°C	5190-3933	5190-3936 and 5190-3937	5 x 5190-3936 and 5 x 5190-3937
SureSelect XT2 Pre-Capture Box 1	Room Temperature	5190-4076	5190-4076	5190-4077
SureSelect XT2 Pre-Capture ILM Module Box 2	–20°C	5190-4461	5190-3940	5190-3942

* See [Table 34](#) through [Table 36](#) for a list of reagents included in each component kit.

† Kits contain reagents to prepare indexed libraries from 16 gDNA samples and to enrich the samples in 1 or 2 hybridization and capture reactions (as appropriate for the specific capture library size and sample pooling format).

‡ Kits contain reagents to prepare indexed libraries from 96 gDNA samples and to enrich the samples in 6 or 12 hybridization and capture reactions (as appropriate for the specific capture library size and sample pooling format).

** Kits contain reagents to prepare indexed libraries from 480 gDNA samples and to enrich the samples in 30 or 60 hybridization and capture reactions (as appropriate for the specific capture library size and sample pooling format).

†† See [Table 44](#) on page 82 through [Table 49](#) on page 87 for index sequence information.

NOTE

SureSelect capture libraries and reagents must be used within one year of receipt.

The contents of each of the component kits listed in [Table 39](#) are described in the tables below.

Table 40 SureSelect XT2 Library Prep Kit, ILM Content-Original Configuration

Kit Component	16 Sample Kit	96 or 480 Sample Kit
SureSelect End Repair Master Mix	tube with orange cap	bottle
SureSelect dA-Tailing Master Mix	tube with yellow cap	bottle
SureSelect Ligation Master Mix	tube with purple cap	tube with purple cap
SureSelect Herculanase II Master Mix	tube with red cap	bottle
XT2 Primer Mix	tube with clear cap	tube with clear cap

Table 41 SureSelect XT2 Pre-Capture Box 1 Content

Kit Component	16 Sample Kit	96 Sample Kit	480 Sample Kit
SureSelect XT2 Binding Buffer	bottle	bottle	bottle
SureSelect XT2 Wash 1	bottle	bottle	bottle
SureSelect XT2 Wash 2	bottle	bottle	bottle

Table 42 SureSelect XT2 Pre-Capture ILM Module Box 2 Content

Kit Component	16 Sample Kit	96 Sample Kit	480 Sample Kit
SureSelect XT2 Blocking Mix	tube with blue cap	tube with blue cap	tube with blue cap
SureSelect XT2 Hybridization Buffer	tube with yellow cap	tube with yellow cap	bottle
SureSelect RNase Block	tube with purple cap	tube with purple cap	tube with purple cap

Table 43 SureSelect XT2 Pre-Capture Index Format-Original Configuration

Kit Component	16 Sample Kit	96 or 480 Sample Kit
SureSelect XT2 Pre-Capture Indexes*	Indexes 1–16 supplied in p/n 5190-3933 in 16 clear-capped tubes	Indexes 1–96 supplied in p/n 5190-3936 and 5190-3937 in 96 clear-capped tubes

* See [Table 44](#) on page 82 through [Table 49](#) on page 87 for index sequences.

Nucleotide Sequences of SureSelect Pre-Capture Indexes-Original Index Configuration

The nucleotide sequence of each SureSelect^{XT2} Pre-Capture Index provided with the original kit configuration is provided in the tables below.

Refer to the sequence information below only if your kit includes p/n 5190-3933 or p/n 5190-3936 and p/n 5190-3937, with indexing primers provided in individual clear-capped tubes.

Each index is 8 nt in length. Sequencing protocols must be run using the third-read protocol that is also used for 9-bp indexes. See [page 71](#) for additional sequencing run setup information.

Table 44 SureSelect^{XT2} Pre-capture Indexes 1-16

Index Number	Sequence
1	AACGTGAT
2	AAACATCG
3	ATGCCTAA
4	AGTGGTCA
5	ACCACTGT
6	ACATTGGC
7	CAGATCTG
8	CATCAAGT
9	CGCTGATC
10	ACAAGCTA
11	CTGTAGCC
12	AGTACAAG
13	AACAACCA
14	AACCGAGA
15	AACGCTTA
16	AAGACGGA

Nucleotide Sequences of SureSelect Pre-Capture Indexes-Original Index Configuration

Table 45 SureSelect^{XT2} Pre-capture Indexes 17-32

Index Number	Sequence
17	AAGGTACA
18	ACACAGAA
19	ACAGCAGA
20	ACCTCCAA
21	ACGCTCGA
22	ACGTATCA
23	ACTATGCA
24	AGAGTCAA
25	AGATCGCA
26	AGCAGGAA
27	AGTCACTA
28	ATCCTGTA
29	ATTGAGGA
30	CAACCACA
31	CAAGACTA
32	CAATGGAA

Table 46 SureSelect^{XT2} Pre-capture Indexes 33-48

Index Number	Sequence
33	CACTTCGA
34	CAGCGTTA
35	CATACCAA
36	CCAGTTCA
37	CCGAAGTA
38	CCGTGAGA
39	CCTCCTGA
40	CGAACTTA
41	CGACTGGA
42	CGCATACA
43	CTCAATGA
44	CTGAGCCA
45	CTGGCATA
46	GAATCTGA
47	GACTAGTA
48	GAGCTGAA

Nucleotide Sequences of SureSelect Pre-Capture Indexes-Original Index Configuration

Table 47 SureSelect^{XT2} Pre-capture Indexes 49-64

Index Number	Sequence
49	GATAGACA
50	GCCACATA
51	GCGAGTAA
52	GCTAACGA
53	GCTCGGTA
54	GGAGAACA
55	GGTGCGAA
56	GTACGCAA
57	GTCGTAGA
58	GTCTGTCA
59	GTGTTCTA
60	TAGGATGA
61	TATCAGCA
62	TCCGTCTA
63	TCTTCACA
64	TGAAGAGA

Table 48 SureSelect^{XT2} Pre-capture Indexes 65-80

Index Number	Sequence
65	TGGAACAA
66	TGGCTTCA
67	TGGTGGTA
68	TTCACGCA
69	AACTCACC
70	AAGAGATC
71	AAGGACAC
72	AATCCGTC
73	AATGTTGC
74	ACACGACC
75	ACAGATTC
76	AGATGTAC
77	AGCACCTC
78	AGCCATGC
79	AGGCTAAC
80	ATAGCGAC

Nucleotide Sequences of SureSelect Pre-Capture Indexes-Original Index Configuration

Table 49 SureSelect^{XT2} Pre-capture Indexes 81-96

Index Number	Sequence
81	ATCATTCC
82	ATTGGCTC
83	CAAGGAGC
84	CACCTTAC
85	CCATCCTC
86	CCGACAAC
87	CCTAATCC
88	CCTCTATC
89	CGACACAC
90	CGGATTGC
91	CTAAGGTC
92	GAACAGGC
93	GACAGTGC
94	GAGTTAGC
95	GATGAATC
96	GCCAAGAC

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In This Book

This guide contains information to run the SureSelect^{XT2} Target Enrichment System for Illumina Multiplexed Sequencing protocol featuring pre-capture indexing.

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